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Insulin-like growth factor-I (IGF-I) is a potent mitogen for breast cancer cells. Binding of IGF-I to its receptor results in activation of the receptor which then phosphorylates the adaptor protein insulin receptor substrate-1 (IRS-1). Our laboratory has shown that phosphorylation of IRS-1 by IGF-I caused enhanced ubiquitin dependent proteasomal degradation of IRS-1. In contrast, interleukin-4 (IL-4) which also phosphorylates IRS-1 does not enhance degradation of IRS-1. The goal of this project is to understand if phosphorylation of different residues on IRS-1 is responsible for the proliferative response mediated by IGF-I compared to the death signal mediated by IL-4. To identify the residues two-dimensional electrophoresis (2D PAGE) of IRS-1 was undertaken. The first task was to isolate phosphorylated ^{32}P-labeled IRS-1 which was successfully completed. Two-dimensional electrophoresis of IRS-1 which is a 180 kDa protein has encountered several technical difficulties. So far, IRS-1 has not been detected either by subjecting immunoprecipitated ^{32}P-labeled IRS-1 to 2D electrophoresis or immunoblotting lysates with IRS-1 antibody after 2D PAGE. In addition, we have not been able to see proteins above 100 kDa on 2D gels either by silver staining or radioisotope detection of ^{32}P or ^{35}S labeled lysates of MCF-7 cells.

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Annual Summary Report

Introduction

Insulin like growth factor-1 (IGF-I) is an important factor which is a potent mitogen for breast cancer cells and the IGF system is a key growth regulatory pathway in breast cancer. Binding of IGF-I to the type I IGF receptor (IGFIR) stimulates a cascade of signaling events leading to cell growth. IGF-I transduces its signal through the adaptor protein insulin receptor substrate-1 (IRS-1). IGF-I causes phosphorylation of IRS-1. The cytokine interleukin-4 (IL-4) shows growth inhibitory effects in breast cancer cells. Our laboratory has shown that IL-4 stimulates programmed cell death and also phosphorylates IRS-1. Thus, two factors with opposing effects on breast cancer cells cause phosphorylation of IRS-1. The objective of this post-doctoral training project is to study the mechanisms by which IRS-1 is coupled with growth stimulatory pathways with the aim of answering the question: How can phosphorylation of IRS-1 be involved in both cell growth and cell death? Our laboratory has shown that IGF-I causes a higher level of phosphorylation of IRS-1 than IL-4 (1). In addition, IGF-I causes acute phosphorylation of IRS-1 with rapid loss of detectable IRS-1 while IL-4 causes sustained phosphorylation of IRS-1. Our laboratory has also shown that phosphorylation by IGF-I causes degradation of IRS-1 by the ubiquitin proteasome pathway (2). The hypothesis is that IRS-1 is differentially phosphorylated by IGF-I and IL-4 in breast cancer cells and that the phosporylation of IRS-1 by IGF-I results in phosphorylation of specific serine/threonine residues which targets IRS-1 for degradation via the ubiquitin pathway. The successful completion of this project will identify those residues of IRS-1 which are phosphorylated by IGF-I and not IL-4 and are, thus, responsible for targeting IRS-1 for degradation and will create a mutant IRS-1 that can neutralize the action of IGF-I or may be change a mitogenic signal to a death signal.

Body

This training grant is looking at the differences in phosphorylation of the adapter protein IRS-1 used by the IGF1R to propagate the mitogenic signal of IGF-I compared to that by IL-4 to promote death of MCF-7 cells. The specific aims are: 1) identifying the tyrosine, serine and threonine residues of IRS-1 phosphorylated by IGF-I and IL-4 and 2) creating mutants of IRS-1 lacking those residues phosphorylated by IGF-I but not IL-4 and testing the effect of mutations on the half-live of IRS-1 and on the biological function in 32D cells which are null for IRS-1 and MCF-7 breast cancer cells. Stable MCF-7 cell lines expressing IRS-1 mutants which have increased half-lives and hence are not degraded rapidly will be established and tested for response to IGF-I mediated growth. IGF-I mediated growth in all cells will be assayed by anchorage-independent and dependent growth.

The experimental approach to identify phosphorylated residues is to use two-dimensional gel electrophoretic separation of undigested and trypsin and staphylococcus V8 protease digested phosphorylated IRS-1 followed by phosphoaminoacid analysis and sequencing of phosphopeptides that differ between IGF-I and IL-4 stimulation of IRS-1.

Task 1 which was the isolation of ^{32}P-labeled IRS-1 from MCF-7 cells treated with IGF-I and IL-4 has been completed. MCF-7 cells were serum deprived for 24 h. Cells were switched to phosphate free medium for 1h and then labeled for 4h with 100 μCi/ml ortho [^{32}P] phosphate. Cells were then treated with 5 nM IGF-I or 50 ng/ml IL-4 for 10 minutes. Cells were lysed and lysates were immunoprecipitated with IRS-1 antibody and separated by SDS-PAGE. Figure 1 shows the immunoprecipitated band of IRS-1 from ^{32}P labeled MCF-7 cells that were untreated (lane labeled SFM) or treated with IGF-I or IL-4. Task 2 was to perform 2D electrophoresis of IRS-1 from cells treated with IGF-I and IL-4. However, several technical difficulties have been encountered during 2D electrophoresis of IRS-1 and the tasks listed in the Statement of Work have not been fully accomplished. We believe the difficulty is due to the large size of IRS-1 (~180 kDa). Initially, immunoprecipitated IRS-1 was subjected to 2D electrophoresis but it was not detected after the second dimension PAGE. Following that I have tried several different approaches to do the 2D electrophoresis. First, cell lysates were subjected to IEF in the first dimension using 11 cm immobilized pH gradient IPG strips and SDS-PAGE in the second dimension. Proteins were then transferred to nitrocellulose followed by immunoblotting with antiphosphotyrosine antibody or IRS-1 antibody. Figure 2 represents one of the several experiments done on 2D electrophoresis of IRS-1. As shown in Figure 2, I have not been able to get a 2D pattern of IRS-1. On the right side of the figure is shown MCF-7 lysates that were applied to a sample application paper and subjected to SDS-PAGE only along with the IPG strip that was first subjected to IEF. The IRS-1 in the lysate subjected to SDS-PAGE was readily detected with IRS-1 antibody while no IRS-1 pattern was detected in the 2D gel. I have used several different phosphotyrosine antibodies to detect phosphorylated IRS-1 including RC-20, PY-20 (both from Transdcution Laboratories), 4G10 (Upstate Biotechnology), p-Tyr100 (Cell Signaling), and two different IRS-1 antibodies. All of these antibodies readily immunoprecipitate and detect IRS-1 on immunoblots following SDS-PAGE of MCF-7 lysates. To ensure that I have the technique working okay, I have performed 2D electrophoresis of another adaptor protein SHC which is also involved in IGF signal transduction. Figure 3 shows the 2D electrophoresis of Shc. Cell lysates were subjected to 2D electrophoresis and then transferred to nitrocellulose and immunoblotted with Shc antibody. As shown in Fig 3, all 3 isoforms of Shc (48, 52 and 66 kDa isoforms) were readily detected after 2D electrophoresis. This suggests that the technique is working. I have also tried different buffers to prepare the cells lysates for 2D PAGE of IRS-1 without any success. I have also tried to vary the conditions during the first dimension isoelectric focusing (IEF). I have also stained the 2D gels with silver stain using the GelCode Color Silver Stain Kit (Pierce, Rockford, IL) and run ^{32}P and ^{35}S labeled lysates on 2D gels and with both detection systems have not been able to see spots above 100 kDa on the gels. Thus, I believe that the high molecular weight of IRS-1 is posing technical difficulties during 2D electrophoresis particularly during the entry of the proteins from the IPG strip to the second dimension acrylamide gel. I am currently using longer IPG strips (18 cm) for the 2D electrophoresis.

Recently, the University of Minnesota has been set up as a site by Thermoquest to test an alternate approach to determining phosphorylation patterns of proteins. I am currently looking into the usefulness of this approach to identify residues on IRS-1 that are

phosphorylated by IGF-I compared to IL-4. The experimental approach would involve digesting the protein with as little purification (perhaps only separating by size or charge) and then determining how much coverage we can get with mass spectrometry. The peptides of interest can then be resolved by the chromatography before the electrospray and the high resolution of the first stage of the mass spectrometry.

In addition to the experiments outlined in the fellowship-training grant I have successfully undertaken another project investigating the mechanism of action of a single chain antibody against the IGF1R (3) and is briefly described in the attached abstract in the appendix on page 12. This work is now near completion and I am currently preparing a manuscript on the *in vitro* effects of the single chain antibody against IGF1R. Furthermore, in collaboration with a collaborator of my postdoctoral mentor, I have analyzed the effects of treatment with the anti-IGF1R single chain antibody on IGF1R signaling molecules in T61 xenograft tumors in mice. A manuscript describing this work is nearly ready to be submitted with me as one of the authors on the paper.

To further enhance my understanding and training as a breast cancer researcher, I attended a workshop on the molecular and morphologic aspects of human cancer titled 'Pathobiology of Cancer' organized by the American Association for Cancer Research in July 2000 at Keystone, CO. I greatly benefited from attending this workshop which provided me with intensive training in the histopathology and biology of neoplasia. In June 2001, I presented a poster on my work with a single chain antibody at the 83rd Annual Meeting of the Endocrine Society.

Key Research Accomplishments:

- Isolation of phosphorylated ^{32}P-labeled IRS-1
- Technical aspects of 2D electrophoresis
- Characterization of the *in vitro* effects of a single-chain antibody against the IGF1R

Reportable Outcomes:

1. I was selected to attend a workshop on the Pathobiology of Cancer at Keystone, CO in July 2000.
2. I presented posters at the 83rd Annual Meeting of the Endocrine Society at Denver, CO from June 19-23, 2001.
3. I have written a review article on the role of IGFs and breast cancer titled "The IGF System and Breast Cancer" for Endocrine-Related Cancer (4) in response to an invitation to my mentor by the editor.
4. I am currently working on a manuscript on my studies on the *in vitro* effects and mechanism of action of a single chain antibody against the IGF1R.

Conclusions

In the coming year I will continue to resolve the technical difficulties of doing 2D electrophoresis on large proteins and continue with the other tasks.

As an alternative approach to answering the original question I am now beginning to work with some IRS-1 truncation mutants. Most of the serine/threonine and tyrosine residues of IRS-1 that are phosphoryalted are near the C-terminus of the protein. We have obtained three truncation mutants of IRS-1 that delete regions of IRS-1 from its C-terminus. Additionally, IRS-1 has a 9 amino acid destruction box consensus motif that targets some proteins for ubiquitination followed by proteasomal degradation. This sequence in human IRS-1 is found at residues 1074-1082. I plan to change the serine residue in the destruction box signal and thereby prevent its phosphorylation and subsequent proteasomal degradation of IRS-1. If the mitogenic ability of IGF-I is due to the rapid turnover of IRS-1, then prolonging the half-live of IRS-1 could impede its mitogenic ability and this would also help us with our long-term goal of uncoupling IGF-I action from the mitogenic pathway.

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Appendix

Figure Legends:

Figure 1: Immunoprecipitation of labeled IRS-1. 70% confluent MCF-7 cells in 10 cm^2 dishes were serum deprived for 24h. Cells were then switched to phosphate-free medium for 1h and then labeled with 100 μCi/ml ortho[^{32}P] phosphate in phosphate-free medium for 4h. Cells were either untreated (lane SFM) or treated with 5 nM IGF-I or 50 ng/ml IL-4 for 10 minutes at 37°C. Cells were lysed in 500 μl TNESV buffer (50 mM Tris, pH 7.4; 1% NP-40; 2 mM EDTA, pH 8.0; 0.1 M NaCl, and 1.84 mg/ml Na orthovanadate, and protease inhibitors). Cellular lysates were centrifuged at 12000g for 20 minutes and supernatants used as lysates. Lysates were precleared with protein A agarose for 30 minutes at 4°C and incubated with IRS-1 antibody overnight at 4°C. Protein A agarose was added and incubated for 4h at 4°C. Immunoprecipitates were washed four times with TNESV buffer and then suspended in 30 μl TNESV and 30 μl of 2X Laemmli sample buffer and subjected to SDS-PAGE. Gel was dried and exposed to film. IRS-1 was seen in all samples and was cut out from the gel and used for other experiments.

Figure 2: 2-dimensional electrophoresis of IRS-1. 100 μg of MCF-7 lysates was applied to an immobilized linear pH 3-10 IPG strip (11 cm) and subjected to IEF using the IPGphor (Amersham Pharmacia) for 25,500 Vh. The strip was then equilibrated with SDS running buffer and subjected to SDS-PAGE in the second dimension. As a control, 40 μg of lysate was applied to a sample application paper and subjected only to SDS-PAGE on the right side of the IPG strip. The proteins were transferred to nitrocellulose and immunoblotted with IRS-1 antibody. While IRS-1 was detected in the lysate applied to a sample application piece and subjected to SDS-PAGE, no spots of IRS-1 were seen on the 2D gel.

Figure 3: 2-dimensional electrophoresis of Shc. MCF-7 lysate was subjected to 2D PAGE as described for Figure 2. The proteins were transferred to nitrocellulose and blotted with a polyclonal antibody against Shc. All 3 Shc isoforms of 46, 52, and 66 kDa were detected after 2D PAGE.

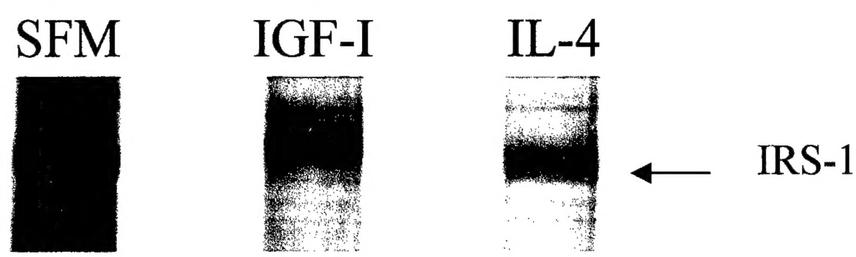

Figure 1: Immunoprecipitation of ^{32}P-labeled IRS-1 from MCF-7 cells

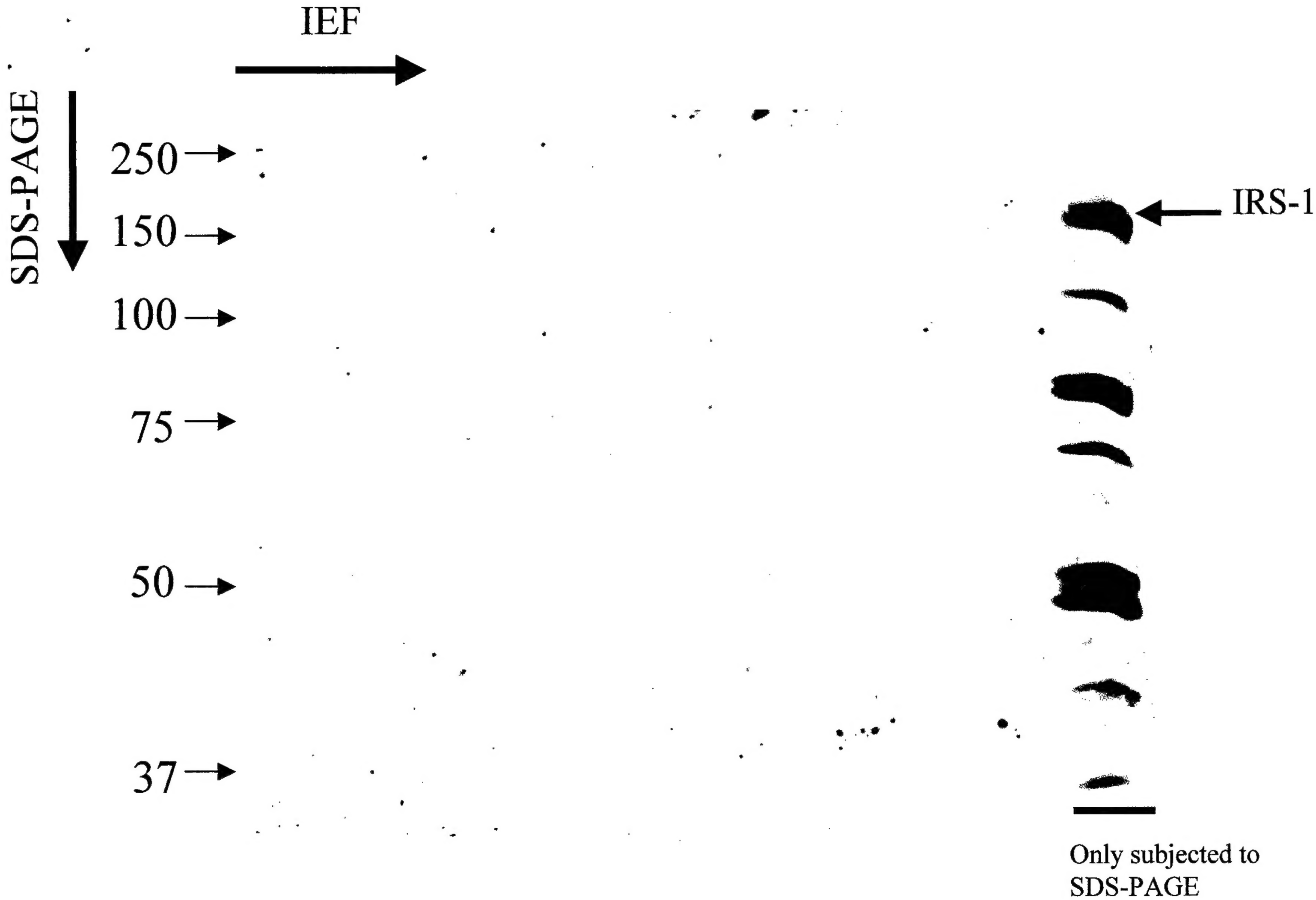

Figure 2: 2D electrophoresis of IRS-1

Figure 3: 2D Electrophoresis of SHC

Copy of abstracts presented at the 83rd Annual meeting of the Endocrine Society

P1-260

A SINGLE CHAIN ANTIBODY AGAINST THE TYPE I INSULIN-LIKE GROWTH FACTOR RECEPTOR (IGF1R) STIMULATES IGF SIGNALING *IN VITRO*

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Insulin-like growth factors (IGFs) stimulate proliferation and survival of breast cancer cells via the type I IGF receptor (IGF1R). We have previously shown that activated IGF1R phosphorylates insulin receptor substrate-1 (IRS-1) in MCF-7 breast cancer cells. Inhibition of IGF1R activation inhibits breast cancer cell proliferation and survival. We have also shown that insulin-like growth factor binding protein-1 (IGFBP-1) inhibits IGF-I, estradiol, and serum stimulated growth of MCF-7 cells. In this study, we examined the effects of a recombinant humanized single chain antibody against the IGF1R (scFv-Fc), which has recently been described (Li *et al.* Cancer Immunol Immunother 49:243, 2000). scFv-Fc partially inhibits growth of MCF-7 cells in athymic mice. We examined the effects of scFv-Fc on key IGF signaling molecules in MCF-7 breast cancer cells. Surprisingly, 250nM scFv-Fc stimulated monolayer growth of MCF-7 cells *in vitro* to levels equivalent to 5nM IGF-I. scFv-Fc and 1nM estradiol resulted in synergistic growth stimulation of MCF-7 cells. Unlike IGFBP-1, scFv-Fc did not inhibit phosphorylation of IRS-1 when cells were preincubated with the antibody for 30 minutes prior to stimulation with IGF-I. Furthermore, scFv-Fc alone enhanced phosphorylation of IRS-1 in MCF-7 cells. scFv-Fc stimulated both MAPK and PI3K pathways in MCF-7 cells. The stimulation of MAPK was similar to that by IGF-I while the activation of PI3K was sustained compared to IGF-I. We have previously shown that IGF-I causes rapid and transient phosphorylation of IRS-1 followed by proteasome mediated degradation of IRS-1. Preliminary evidence suggests that scFv-Fc does not enhance the degradation of IRS-1 even over a 24-hour period. Our data suggest that *in vitro* scFv-Fc behaves as an agonist of the IGF1R and activates key IGF signaling molecules. These results suggest that analysis of single chain antibody efficacy must be approached with caution. Under certain conditions, such antibodies may act as full agonists of their intended receptor targets. Inhibitory and stimulatory activity may be dependent on cell surface receptor density and kinetics of receptor phosphorylation.

obtained even at low concentrations and can not be substituted for by estrogen alone, appears to be necessary in order to induce re-entry of the MCF-7 into the cell cycle. We have now studied such IGF-I specific events in detail by looking at the subcellular localization and the activity of the cyclin D1 / cdk4 complex in the presence and absence of IGF-I. Although the complex is formed after IGF-I as well as after estrogen addition to the arrested cells, it only shows its specific activity when IGF-I is present.

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INSULIN RECEPTOR SUBSTRATE-2 (IRS-2) ACTIVATION IN MCF-7 BREAST CANCER CELLS IS ASSOCIATED WITH CELL MOTILITY

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The insulin-like growth factors (IGFs) stimulate cell proliferation and motility in some breast cancer cell lines. Interaction of IGF-I with its receptor (IGF1R) results in activation of specific adaptor proteins. In the MCF-7L breast cancer cell line, IGF-I activates IRS-1 and enhanced cell proliferation but did not stimulate cell motility. In contrast, in the MDA-231 BO breast cancer cell line, IGF-I activated IRS-2 mediated cell motility without stimulation of proliferation. These data suggest that proliferation and motility may be mediated by distinct IRS proteins in breast cancer cells. Other strains of MCF-7 behave differently in response to IGF-I. Both MCF-7L and MCF-7ATCC proliferated in response to 5nM IGF-I and 1nM estradiol. However, only MCF-7ATCC had enhanced motility in response to IGF-I. The purpose of this study was to determine if differences in IRS activation accounts for these different IGF-mediated phenotypes. In both cell lines, IGF-I treatment resulted in phosphorylation of a 185kDa protein. Immunoblots showed that IRS-1 and IRS-2 levels were equivalent between the two cell lines. Immunoprecipitation with IRS-1 antibody followed by anti-phosphotyrosine blotting showed that IRS-1 was equally activated by IGF-I in both cell lines. In contrast, IRS-2 immunoprecipitation showed that MCF-7ATCC had increased IRS-2 phosphorylation compared to MCF-7L. Both MCF-7 cell lines had similar activation of MAPK and PI3K after IGF-I treatment. These data suggest that the biological effects of IGF-I are mediated by activation of specific adaptor proteins downstream of IGFR1. Activation of IRS-2 correlates with IGF-I mediated cell motility while IRS-1 activation is required for proliferation.

previous studies using Caco-2 cells demonstrated that overexpression (S) of endogenous IGFBP-2 in Caco-2 cells was associated with increased cell proliferation and tumorigenesis compared to IGFBP-2 antisense (AS), mock (M) transfected and wild type (WT) cells. The aim of the present study was to investigate the effect of IGFBP-2 on apoptosis induction of Caco-2 cells. Apoptosis was analysed by Western immunoblot (IB) for caspase-8 and immunocytochemistry (ICC) for cytokeratin 18. For the ICC the M-30 antibody was used that recognises a caspase cleave epitope of cytokeratin 18, which is considered as an indicator of apoptosis in epithelial cells. The IGFBP-2-S, AS, M and WT Caco-2 cells were grown to confluence. At day 4 cells were rinsed with serum free medium and incubated for 24 and 48 hr. Total protein was then extracted and analysed by SDS-PAGE and IB with anti caspase-8 antiserum. For ICC analysis cells were prepared in tissue culture slides and treated as before. IB analysis showed that caspase-8 was activated after 24 hr in M cells by 150% compared to wild-type cells. IGFBP-2 AS cells had a further 93% increase in caspase-8 activation compared to M cells. In contrast, in IGFBP-2-S cells no activation of caspase-8 was seen. ICC for M-30 antibody gave similar results with those from caspase-8 IB. Wild-type cells had almost no staining for M-30 antibody. M-cells had some positive cells (1 cell/optical field), whereas IGFBP-2-AS cells had more stained cells (2.5 cells/optical field). Again, IGFBP-2-S cells were negative for M-30. Suppression of endogenous IGFBP-2 in antisense transfected Caco-2 cells increased apoptosis compared to cells expressing IGFBP-2. These results indicate that IGFBP-2 is an important regulator of colon cancer cell growth and programmed cell death.

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COLON CANCER GROWTH AND METASTASIS IS DEPENDENT ON CIRCULATING IGF-1 LEVELS

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Slight elevations in serum levels of insulin-like growth factor-1 (IGF-1) were shown to correlate with the risk for prostate, breast, colon and lung cancer. The aim of our study was to verify the role of serum IGF-1 levels in the process of stimulating tumor growth and metastasis. For this purpose, we used a mouse model of liver metastasis from the colon by attaching colon 38-adenocarcinoma tissue fragments on the surface of the cecum. Tumor tissue was attached to the control and liver-specific IGF-1-deficient mice (LID) in which serum IGF-1 levels are 25% of control levels. Attachment of tumor tissue